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Bioactive compound loaded stable silver nanoparticle synthesis from microwave irradiated aqueous extracellular leaf extracts of *Naringi crenulata* and its wound healing activity in experimental rat model

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ABSTRACT

An efficient and eco-friendly protocol for the synthesis of bioactive silver nanoparticles was developed using *Naringi crenulata* leaf extracts via microwave irradiation method. Silver nanoparticles were synthesized by treating *N. crenulata* leaf extracts with 1 mM of aqueous silver nitrate solution. An effective bioactive compound such as alkaloids, phenols, saponins and quinines present in the *N. crenulata* reduces the Ag^+ into Ag^0. The synthesized silver nanoparticles were monitored by UV–vis spectrophotometer and further characterized by X-ray diffraction (XRD), Fourier Transform Infra Red (FTIR), Energy-dispersive X-ray spectroscopy (EDX) and field emission scanning electron microscopy (FESEM). UV–vis spectroscopy showed maximum absorbance at 390 nm due to surface plasmon resonance of AgNPs. From FESEM results, an average crystal size of the synthesized nanoparticle was 72–98 nm. FT-IR results showed sharp absorption peaks and they were assigned to phosphine, alkyl halides and sulfonate groups. Silver nanoparticles synthesized were generally found to be spherical and cubic shape. Topical application of ointment prepared from silver nanoparticles of *N. crenulata* were formulated and evaluated *in vivo* using the excision wound healing model on Wistar albino rats. The measurement of the wound areas was performed on 3rd, 6th, 9th, 12th and 15th days and the percentage of wound closures was calculated accordingly. By the 15th day, the ointment base containing 5% (w/w) of silver nanoparticles showed 100% wound healing activity compared with that of the reference as well as control bases. The results strongly suggested that the batch C ointment containing silver nanaoparticles synthesized from the leaf extracts of *N. crenulata* was found to be very effective in wound repair and encourages harnessing the potentials of the plant biomolecules loaded silver nanoparticle in the treatment of tropical diseases including wound healing.

1. Introduction

Biosynthesis of stable nanoparticles has received considerable attention due to the unique physicochemical characteristics of nanoparticles, including catalytic activity, optical properties, electronic properties, antibacterial properties and magnetic properties (Bar et al., 2009; Tuutijarvi et al., 2009; Rassaei et al., 2008). Specific surface area is relevant to catalytic activity and other related properties such as antimicrobial activity of AgNPs (Bae et al., 2010; Gurunathan et al., 2009; Pal et al., 2007). Silver nanoparticle has wide range of applications in spectrally selective coatings for solar energy absorption, optical receptors, bio-labelling intercalation materials for electrical batteries, filters, antimicrobial agents and sensors (Smitha et al., 2008; Mohanpuria et al., 2008). Nanoparticles can be synthesized by physical, chemical and biological methods. Non-biological methods of nanoparticle synthesis used to cause accumulation of toxic and non-eco-friendly by-products. The development of biological approaches for the synthesis of nanoparticles by intracellular or extracellular reduction is essential for the production of ecofriendly and non-toxic nanoparticles. Several biological systems including plants, bacteria, fungi and algae have been used for the synthesis of nanoparticles (Kalimuthu et al., 2008; Kowshik et al., 2003; Shahverdi et al., 2007; Sanghi and Verma, 2009). Among the biological methods, plant extract based green synthesis of nanoparticles is the best eco-friendly alternative tool to available conventional chemical and physical methods (Sreeram et al., 2008; Willner et al., 2006).

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Plants provide a better platform for nanoparticle synthesis. Silver has a greater affinity towards sulfur or phosphorus-containing biomolecules present in the cells of plant leaves. Hence, sulfur or phosphorous-containing proteins in the membrane or inside the cells are considered to be the preferential sites for silver nanoparticle binding (McDonnell and Russell, 1999). The plant material based production of silver nanoparticles has wide range of application in medical industries in food processing industries (Tankhiwale and Bajpai, 2010) and in textile industries. In medicines, silver and silver nanoparticles deliver extensive application including skin ointments and creams containing silver to infection of burns and open wounds (Duran et al., 2005). Plants have been used for low-cost, energy-efficient and non-toxic production of metallic nanoparticles. Silver nanoparticle synthesis have been reported by Rastogi and Arunachalam (2011) in *Allium* sativam, Sheny et al. (2011) in *Anacardium occidentale*, and Chandran et al. (2006) in *Aleo vera* plant extract. Also the formation of gold and silver nanoparticles was reported for the first time by using living plants (Gardea-Torresdey et al., 2002; Jose-yacamann et al., 2003).

Wound healing process occurs in several steps that involve blood coagulation, inflammation, cell proliferation, remodelling of connective tissue and acquisition of wound strength (Suresh Reddy et al., 2002). The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conductive to tissue repair and regeneration (Bowler et al., 2001). Physiologically, wound healing is mainly depends on interaction between a variety of cells, biochemical mediators and extra cellular matrix molecules (Singer and Clark, 1999). Development of drugs/medicines to treat wounds has been conducted for several years. At present different medicinal plants included into the healthcare systems. Among them *Naringi crenulata* plant crude extract possess skin lighting and cosmetic activities or exhibit antimicrobial properties which are beneficial uses in skin wound repair mechanisms.

N. crenulata (Roxb.) belonging to the family Rutaceae occurs naturally in Southeastern Asia. Its synonym is *Hesperethusa crenulata* (Roxb.) M. Roem or *Limonia crenulata* Roxb. Traditionally, *N. crenulata* have long been valued for their medicinal and cosmetic property. The important ingredients present in the *N. crenulata* leaf extracts are octadecanoic acid, T-tetradecenal (*Z*) and *n*-hexadecanoaic acid protein, lipid, carbohydrate, reducing sugar, phenol, tannin, flavonoid, saponin, and alkaloids (Sampathkumar and Ramakrishnan, 2011). In survey of historical accounts on old folk medicines all parts of *N. crenulata* viz. root, stem, bark, leaf and fruit are used in several ailments. The leaves of *N. crenulata* used in the treatment of epilepsy (Ramani et al., 2010). Root is used as remedy for cobra bite (Sekhar et al., 2011), stem powder serve as anti-aging (Kanlayavattanakul et al., 2009) and bark is used to treat puerperal fever (Murty et al., 2010). Although various parts of *N. crenulata* plant are being used for treatment, skin lightening agent, Arbutin was found to be high in leaf extracts (Lourith et al., 2010). Different medicinal plants extracts have been widely used for the treatment of wound healing process in the recent past. Hence an effective and powerful wound healing agents from medicinal plants are not available at present. Therefore, the investigation for the development of efficient wound healing agents from medicinal plants has become a thrust area of current research.

Earlier studies have mostly investigated the effect of crude plant extracts on wound healing process (Somboonwong et al., 2012). To the best of our knowledge, there were no reports on wound healing activity using neither crude extracts nor the silver nanoparticles synthesized using *N. crenulata* plant extracts so far. It is reported that microwave irradiation based nanoparticle synthesis has certain advantages including short duration, small size particles with narrow distribution and high purity over usual methods. In view of above, it is decided to use microwave irradiation method for the synthesis of stable silver nanoparticles using aqueous extracellular leaf extracts of *N. crenulata*. The present study is mainly focused to extracellular synthesis of bioactive compound loaded silver nanoparticles using *N. crenulata* plant leaf extracts by microwave irradiation method and to evaluate its wound healing activity in wistar albino rats.

2. Materials and methods

2.1. Preparation of leaf extracts

Fresh leaves of *N. crenulata* were collected from ABS (Altogether Botanical Species) Medicinal Plants Garden, Karipatti, Salem, Tamilnadu, India. The collected fresh leaves were surface cleaned with running tap water, followed by distilled water. Biosynthesis of silver nanoparticles was carried out by microwave irradiation method.

2.2. Microwave irradiation method

Fresh leaf samples (10 g) were finely chopped and mixed with 50 ml of deionized water. The mixture was boiled in microwave oven for 10 min. After boiling, the samples were allowed to cool at room temperature and the extracts filtered with Whatman No.1 filter paper.

2.3. Synthesis of silver nanoparticles

About 25 ml of aqueous extract of *N. crenulata* from microwave method was added to the 225 ml aqueous $AgNO_3$ (1 mM) solution in Erlenmeyer flask. This was mixed thoroughly and then the reaction mixture was manually shaked well and kept under dark conditions until the colour change was noticed. The reaction progress for the formation of AgNPs was monitored by visual colour change and UV–vis spectral scanning in the range of 300–700 nm.

2.4. Characterization of silver nanoparticles

Synthesized silver nanoparticles was confirmed by sampling the reaction mixture at regular intervals and absorption maxima was scanned by UV–vis spectra, at the wavelength of 300–700 nm in Systronics 2350 double beam spectrophotometer. In X-ray diffraction analysis, crystalline metallic silver was examined by coating dried nanoparticles on XRD grid using Rigaku miniflex II. X-ray diffraction (XRD) measurement of film of the biologically synthesized silver nanoparticles solution cast onto glass slide was performed at voltage 30 kV with Cu $k(\alpha)$ radiation of 1.54187 nm wavelength. The scanning region of 2θ ranges from 20° to 80°. Purified AgNPs in the form of powder were analysed using FT-IR spectral measurements. The measurements were carried out on a Bruker tensor 27 instrument. The samples were mixed with KBr to make a pellet and it was placed into the sample holder. The spectrum was recorded at a resolution of 4 cm^{-1}. The shape and size of synthesized AgNP was examined to record micrograph image using field emission scanning electron microscopy (FESEM, CARL ZEISS) and these images were operated at 5 kV. The presence of elemental silver was determined in order to check the surface inter-atomic distribution using energy dispersive X-ray (EDX).

2.5. Formulation of the ointment

A simple ointment base as per Indian pharmacopeia was prepared by fusion method. Three batches of the simple ointment (Batch A, B, and C) were prepared and used in this study. Batch

A ointment was prepared with neither the nanoparticle nor the standard drug used as control. Batch B ointment was formulated to contain betadine (5% w/w) while Batch C ointment contained silver nanoparticles (5% w/w) synthesized using *N. crenulata.* The required quantities of the nanoparticles or the standard antibiotics (betadine) was weighed, added to the molten ointment base and then homogenized by trituration.

2.6. *In vivo* wound healing activity

2.6.1. Experimental animals

Wistar male albino rats (120–150 g) were used for *in vivo* studies. The animals were randomly allocated into three groups of four animals each for the excision wound experimental model. The rats were used after acclimatization period of 7 days to the laboratory environment. All animals were kept in polypropylene cages and maintained under standard housing conditions (room temperature 23 ± 2 °C, humidity 50 ± 5 °C with 12:12 light: dark cycles). Food was provided in the form of dry pellet and water. The animals experimental study was conducted after obtained the approval of Institutional Animal Ethics Committee, Mother Terasa Women's University, Kodaikanal (IAEC Approval No. 001/BT/MTWU/IAEC/0209/2013/01 and CPCSEA Registration No. 933/PO/c06/CPCSEA).

2.6.2. Excision of wound model

Animals were anaesthetized with diethyl ether and hairs were removed by shaving the back of all rats. One-excision wound was inflicted by cutting away a 500 mm^2 full thickness of skin from the depilated area and marked using marker. The wound was left undressed to the open environment and no local or systemic antimicrobial agents were used to monitor wound contraction. The rats were distributed in groups randomly and each rat was placed in an individual cage. The wounds of the animal were treated topically depending on the group. Group I considered as negative control and was treated with the blank ointment formulation (batch A). Group II was treated with the standard betadine ointment (batch B). Group III was treated with silver nanoparticle ointment (batch C). The measurement of the wound area of the excision wound model was made on 3rd, 6th, 9th, 12th and 15th day using translucent graph paper. The measurement of wound on graph paper was expressed as unit (mm^2). Wound contraction was expressed as percentage reduction of original wound size.

2.7. Statistical analysis

The experimental results were expressed as the mean ± standard error of mean (SEM) and the data were analyzed using one-way analysis of variance (ANOVA) followed by Student *t* test. Differences in mean between paired observations were considered statistically significance when *p*-values were <0.05.

3. Results and discussion

3.1. Biosynthesis of silver nanoparticles

The addition of *N. crenulata* leaf extracts to silver nitrate solution (1 mM) resulted in colour change of reaction mixture from yellow to brown due to the production of silver nanoparticles after 24 h of incubation in the dark conditions (Fig. 1). It is renowned that AgNPs exhibit dark brown colours, depending on the intensity and the size of nanoparticles; the colours arise due to the excitation of surface plasmon resonance (SPR) of the AgNPs (Prakash et al., 2013). The SPR absorbance was extremely sensitive to the nature, size and shape of the particles formed, their inter particle distances and the surrounding media. A variation in the biological material concentration is known to influence NP synthesis (Pimprikar et al., 2009).

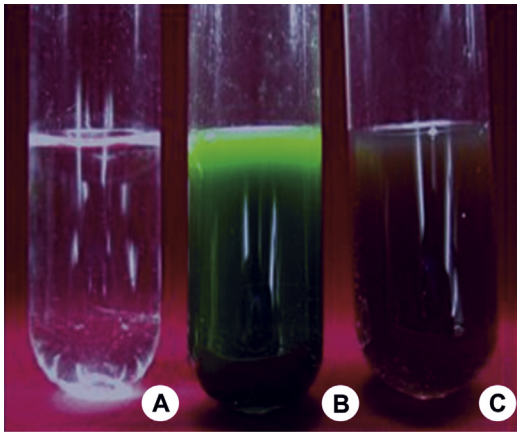

Fig. 1. Visual observations of reaction mixtures: (A) $AgNO_3$, (B) plant extract, (C) synthesized $AgNO_3$ (microwave irradiation method).

3.2. UV–vis spectral study

UV–vis spectroscopy is a significant technique to authenticate the formation and stability of AgNPs in aqueous solution. UV–vis spectra were recorded for the mixture of *N. crenulata* leaf extract and 1 mM silver nitrate solution at various time intervals (0, 1 h, 2 h, 3 h, 4 h, 5 h and 24 h). The colour changes arise because of the excitation of surface plasmon resonance for the synthesized silver nanoparticles (Mulvaney, 1996). The silver nitrate and leaf extract reaction mixture from microwave method exhibits the peak at 390 nm (Fig. 2). The increase in intensity could be due to increasing number of nanoparticles formed as a result of reduction of silver ions present in the aqueous solution. Earlier report stated that maximum absorbance occurred due to presence of silver particle (Sathishkumar et al., 2009). The peak area increased with the increase in reaction time. The possible reason for this observation could be due to the bio-reduction of silver ion by the biomolecules present in the leaf extracts (Huang et al., 2007). The control solutions of *N. crenulata* leaf extract or 1 mM $AgNO_3$ neither developed the characteristic brown colors nor did they display the characteristic peaks. These results indicated that abiotic reduction of $AgNO_3$ did not occur under the reaction conditions. Similar results were also reported recently (Jagtap and Bapat, 2013).

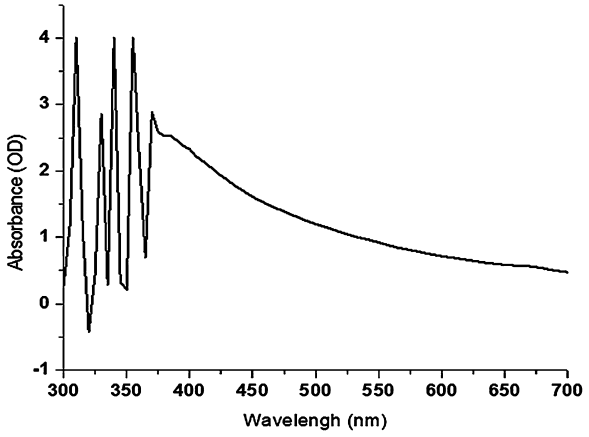

Fig. 2. UV–vis spectrum of Ag nanoparticles by microwave method.

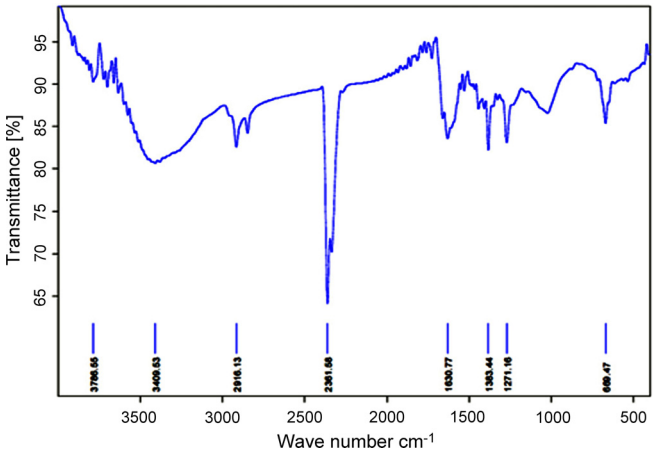

Fig. 3. FTIR pattern of biosynthesized silver nanoparticles by microwave method.

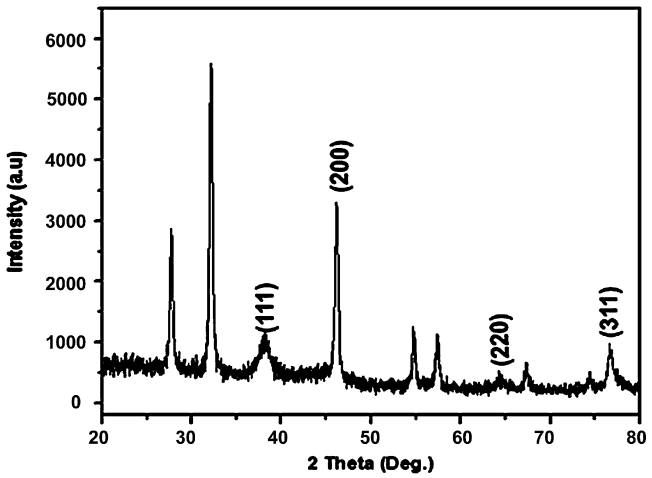

Fig. 4. XRD pattern of silver nanoparticles synthesized using *Naringi crenulata* by microwave method.

3.3. Fourier-transform FT-IR study

The FT-IR spectroscopy measurements were carried out to identify the possible biomolecules present in the leaf extracts that bound specifically on the silver surface. The biologically synthesized silver nanoparticles obtained from microwave irradiated method were mixed with potassium bromide to make a pellet. The FT-IR spectra were collected at resolution of 4 cm^{-1} in the transmission mode (4000–400 cm^{-1}) using BRUKER TENSOR 27. The IR spectrum of silver nanoparticles obtained from microwave irradiated method showed the absorption peaks at 3786.5, 3409.5, 2916.1, 2361.5, 1630.77, 1383.44, 1271.1 and 669.4 cm^{-1} (Fig. 3). The bands appeared at 2361.4, 1383.9 and 670 cm^{-1} are due to the presence of phosphines(P—H), sulfonates(S=O) and alky halides(C—Br) stretching, respectively. The bands at 1650–1550 cm^{-1} were characteristic of amide groups (Caruso et al., 1998). The amide band arises either as a result of stretch mode of the carbonyl group coupled to the amide linkage or N—H stretching mode of vibration in the amide linkage. As mentioned earlier, the *N. crenulata* leaves are very rich source of bioactive compounds such as tannins, phenols, flavonoids, saponin, quinine, protein, lipid and triterpenoid, alkaloids. The proteins can easily bind to silver nanoparticles through either free amine groups or cystein residues in the proteins which ultimately stabilizes the silver nanoparticles (Gole et al., 2001). Biological components are known to interact with metal salts via these functional groups and mediate their reduction to nanoparticles. The IR spectroscopic studies confirmed that phosphines and sulfonates possess strong binding ability and acting as capping agent to provide stability to the silver nanoparticles. A similar observation is noticed in biological synthesis of AgNPs using leaf extracts of *Mimusops elengi* (Prakash et al., 2013) and *Ocimum sanctum* (Subba Rao et al., 2013).

3.4. X-ray diffraction analysis

X-ray diffraction studies were performed to confirm the crystalline structure of the synthesized silver nanoparticles. XRD spectrum of silver nanoparticles obtained by microwave irradiated method revealed diffraction peaks at 32.28°, 46.28°, 54.83°, 67.47° and 76.69° (Fig. 4). The average crystal size of the silver nanoparticles formed in the bioreduction process was determined by full width at half maximum (FWHM) data along with Debye Scherrer's equation $D = k\lambda/\beta \cos\theta$ ($k = 0.89$; $\lambda = 1.5406$ Å). The average crystal size estimated was approximately 83 nm. Unidentified crystalline peaks (32.28°, 46.28°, 54.83°, 67.47° and 76.69°) were also apparent in many works in which the XRD pattern includes the relevant 2θ range (Kumar and Yadav, 2009; Jeeva et al., 2014). Appearances of these peaks were due to the presence of phytochemical compounds in the leaf extracts.

3.5. FESEM and EDX study

FESEM images were measured and topographical analysis was performed based on the surface study. The FESEM studies provide

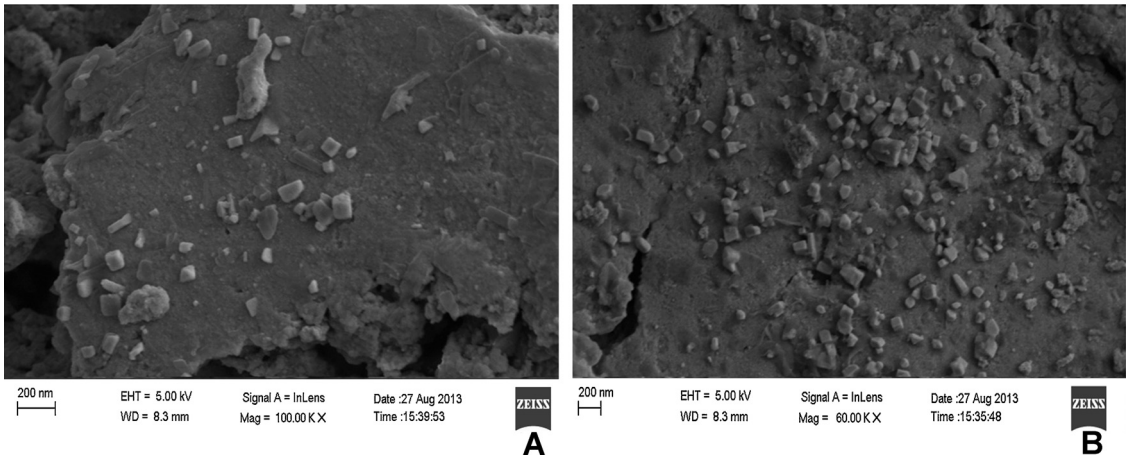

Fig. 5. Topographical results of AgNps confirming the cubical (A) and spherical (B) shaped from FE-SEM analysis of *Naringi crenulata* (Roxb.).

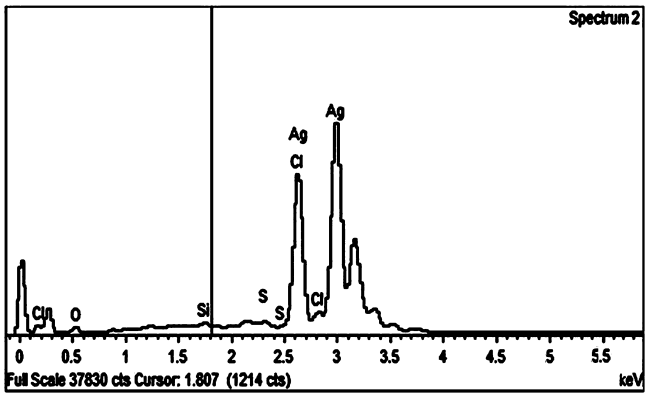

Fig. 6. Energy dispersive X-ray spectrum (EDX) of metallic AgNPs.

the information on the morphology and size of the synthesized silver nanoparticles. According to the FESEM micrograph, the morphology of the silver nanoparticle was observed spherical and cubic structures. The sizes of the cubic Ag nanoparticle were found to be in the range of 72–98 nm. Most of the nanoparticles aggregated and only a few of them were scattered, as observed under FESEM. The EDX spectrum of synthesized silver nanoparticles clearly exhibited the absence of elemental nitrogen and oxygen peaks and the presence of elemental silver metal (Fig. 5). The sharp signal peak of silver strongly confirmed the reduction of silver nitrate to silver nanoparticles. Strong signals from the Ag atoms in the nanoparticles were observed and signals from S, O and Cl atoms were also recorded. The signals were likely due to X-ray emission from carbohydrates/proteins/enzymes present in the cell wall of the biomass (Mishra et al., 2010). Xu and Kall (2002) demonstrated that the shape of metal nanoparticles was considerably changed their optical and electronic properties due to the presence of various bioactive molecules. The EDX attachment present with the FESEM is known to provide information on the biochemical analysis of the fields that are being investigated or the composition at specific locations (spot EDX). In the presence study, the metallic silver nanocrystals showed strong absorption spectra in the range 2.5–4 keV (Fig. 6). Similar results were also reported earlier by Gardea-Torresdey et al. (2003). Recently, Jagtap and Bapat (2013) obtained formation of irregular shape silver nanoparticles at 2.983 keV by using *Artocarpus heterophyllus* seed extracts and Vijayakumar et al. (2013) reported the square shape nanocrystals in the range 2–4 keV using *Artemisia nilagirica* leaf extracts.

3.6. Evaluation of wound healing activity

The wound healing activity of ointment base formulated with synthesized silver nanoparticles from *N. crenulata* was examined on excision wound models with control and standard ointment base. The measurements of the progress of the wound healing induced by ointment base (control), betadine (standard) and nanoparticle in ointment base were shown in Table 1. No death was observed for any of the rats in the study group and there were no remarkable changes in animal behaviour. It is very interesting to note that, topical application of the silver nanoparticles incorporated ointment base (Batch C) showed a significant ($p < 0.05$) rate of wound healing activity in rats than that of control and standard (Fig. 7). The wound closure time was lesser, as well as the percentage of

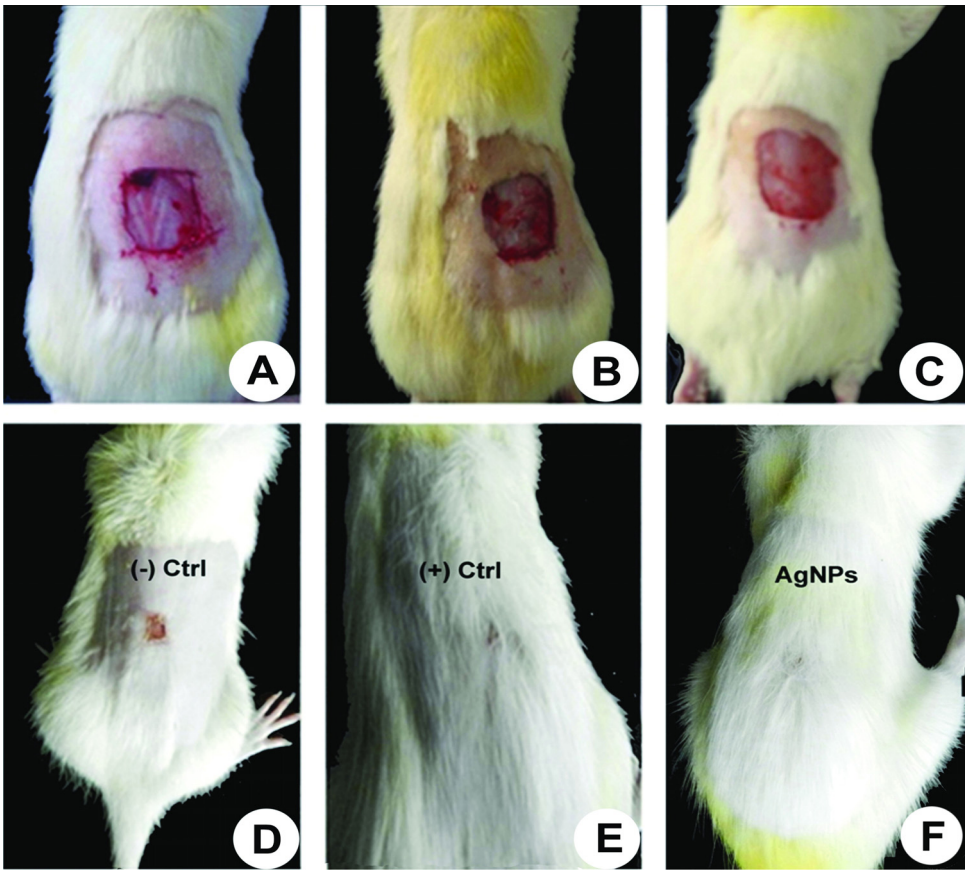

Fig. 7. Progress of cutaneous wound healing in negative-control rats untreated ((A) and (D)), positive control rats treated with betadine ointment ((B) and (E)) and experimental rats treated with *Naringi crenulata* biomolecules loaded AgNp based ointments((C) and (F)). Wounds of the dorsal skin at before and after treatment without ((A) and (D)), and with ((B), (C), (E) and (F)) ointments.

Table 1
Effect of externally applied phytosynthesized silver nanoparticles based ointment on excision wound in experimental rat model.

Animal treatment groups*	Wound contraction (mm^2) on day ± SE and percentage of wound contraction					
	0 day	3 days	6 days	9 days	12 days	15 days
Group I	574.5 ± 3.61	465.4 ± 6.8	395.3 ± 6.3	315.6 ± 2.1	276.2 ± 3.2	185.3 ± 2.1
Group II	524.6 ± 2.31	410.5 ± 4.6	306.4 ± 5.1	129.4 ± 2.3	64.2 ± 1.5*	8.1 ± 3.4*
Group III	513.3 ± 5.1	387.6 ± 5.9	299.1 ± 2.4	143.5 ± 3.4	38.4 ± 1.14*	0.0 ± 0.0*

* The values are mean ± SEM ($n = 4$) statistically significant difference in comparison with control group: $p < 0.05$.

wound area diameter was much more with the silver nanoparticle treated group (15 days for 100% contraction). The least wound healing activity was noticed in the control than standard group. The wound contraction is the process of mobilizing healthy skin surrounding wound area to cover the denuded area and this centripetal movement of wound margin is believed to be due to the activity of myofibroblast. Recent studies have shown that phytochemical constituents like tannins, phenols, flavonoids, saponin, triterpenoid, alkaloids are known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelialization. Medicinal plant based ointments used in folk medicine were reported to have beneficial effect in wound care and healing (MacKay and Miller, 2003; Odimegwu et al., 2008). As wound has little breaking strength in the beginning, that increases rapidly during healing due to the synthesis of collagen and formation of intra and intermolecular cross linking. Here, an increase in skin breaking strength of the animals treated with the AgNPs based ointment formulations explained that the active biomolecules present in it were assisted in the enhanced synthesis of aldehyde groups of collagen fibres for cross linkage of the skin in the rats. The present wound healing activity results showed that *N. crenulata* bioactive molecules loaded AgNps based ointment was found to be effective in wound healing repair mechanism and encourages the harnessing of the AgNPs in the formulation of AgNPs based dermatological ointment for commercial applications.

4. Conclusion

An efficient method was developed to synthesize biomolecules loaded AgNPs using aqueous *Naringi crenulata* leaf extracts that acted both as reducing and capping agents. Silver nanoparticles were synthesized by microwave irradiation method and characterized by UV–vis spectroscopy, XRD, FTIR, FESEM equipped with EDX. FTIR results confirmed the presence of various phytochemicals viz., Phosphines, Sulfonates, Amides and Alky halides in the leaf extracts of *N. crenulata*. The presence of various phytochemical constituents in leaf extracts had reduced the silver ion into metallic silver nanoparticles. The present results clearly showed that biomolecules loaded AgNPs were significantly promoted the wound healing process in rats due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelialization. The results also indicated that the biomolecules coated silver nanoparticles based ointment was found to be efficient to modulate the cytokine activity during wound healing process. To the best of our knowledge this is the first report on synthesis and characterization of AgNPs using leaf extracts of *N. crenulata* and its efficacy on wound healing potential in rats.

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